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Crystallization and preliminary X-ray diffraction studies of bleomycin-binding protein encoded on the transposon Tn*5*

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A bleomycin-binding protein, designated BLMT, encoded on the transposon Tn*5* was crystallized using the vapour-diffusion method in a form suitable for X-ray diffraction analysis. Crystals were grown at pH 6.5 in 0.1 *M* sodium cacodylate and 0.2 *M* calcium acetate, using 25% PEG 6000 as a precipitant. They belong to the orthorhombic system, space group $C222_1$, with unit-cell dimensions a = 81.56, b = 85.25, c = 78.91 Å and one dimer in the asymmetric unit. The diffraction intensity data was collected on beamline 18B of the Photon Factory to 2.0 Å resolution with a merging R value of 0.052. The diffraction data set is 91% complete.

1. Introduction

Bleomycin (Bm), a glycopeptide antibiotic produced by *Streptomyces verticillus* (Umezawa, 1974), is an important antitumour agent which causes cell death as a result of multiple strand scissions by direct interaction with the tumour-cell DNA.

The transposon Tn*5* was originally found as a kanamycin-resistant determinant present on an R factor of *Klebsiella* (Berg *et al.*, 1975). Later, the genes encoding antibiotic resistance determinants to streptomycin (Mazodier *et al.*, 1982) and Bm (Genilloud *et al.*, 1984; Mazodier *et al.*, 1985) were reported to be adjacent to the kanamycin-resistance gene. The nucleotide sequence of the Bm-resistance gene, designated *ble*, suggests that *ble* encodes a protein consisting of 126 amino acids with a molecular weight of 14058 Da (Mazodier *et al.*, 1985).

We have cloned and sequenced two independent Bm-resistance genes, designated *blmA* and *blmB*, from the chromosomal DNA of Bm-producing *S. verticillus* (Sugiyama *et al.*, 1994). The *blmA* gene product, designated BLMA, was physicochemically characterized and shown to be a protein consisting of 122 amino acids, with a strong affinity for Bm (Sugiyama *et al.*, 1995).

Streptoalloteichus hindustanus produces tallysomycin, a Bm analogue. A Bm-resistance gene from this organism, designated Sh*ble*, has been cloned and sequenced (Gatignol *et al.*, 1988; Drocourt *et al.*, 1990).

Almost all strains of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated in Hiroshima University Hospital, Japan, were resistant to Bm. We have cloned and sequenced a Bm-resistant gene, designated *blmS*, from the MRSA's chromosomal DNA, demonstrating that the gene is identical to that located on the a staphylococcal plasmid pUB110 (Bhuiyan *et al.*, 1995). The Sh*ble* and *blmS* gene products were bleomycin-binding proteins like BLMA (Gatignol *et al.*, 1988; Sugiyama *et al.*, 1995).

The mechanism by which the *ble* confers Bm resistance is unknown. Three possible models include: reduced Bm permeability into the cell, inactivation of Bm or repair of Bm DNA lesions (Blot *et al.*, 1993). It has been shown that the *ble* gene product induces expression of an alkylation-inducible gene, *aidC*, and that both the *aidC* gene product and DNA polymerase I encoded by *polA* are required for *ble* to confer Bm resistance (Blot *et al.*, 1993). In contrast, the Sh*ble* protein does not require the *aidC* and *polA* genes to confer Bm resistance (Blot *et al.*, 1993). Thus, it has been suggested that the mechanism of action by which *ble* confers Bm resistance arises from repair of Bm DNA lesions. In addition, the expression of *ble* has been reported to give a survival advantage to the bacterial host (Blot *et al.*, 1991).

We have found that the *ble* gene product, designated BLMT, is a protein with a strong affinity for Bm (Kumagai *et al.*, 1999). In fact, when incubated with BLMT, the antibacterial and DNA-cleaving activities of Bm disappeared. These characteristics suggest that BLMT has two functions, conferring survival advantage and Bm resistance to the host cells.

The three-dimensional structure of Sh*ble* protein has been determined at 2.3 Å resolution (Dumas *et al.*, 1994). We have also crystallized BLMA (Kumagai *et al.*, 1998) and determined the crystal structure of BLMA at 1.5 Å high resolution (Kawano *et al.*, 1999), demonstrating that the protein forms a dimer structure.

Although BLMT has similar physicochemical characteristics to BLMA (Kumagai *et al.*,

Table 1
Data-collection statistics.

Resolution (Å)	Number of reflections measured	Number of independent reflections	$I/\sigma(I)$	R_{merge}	Completeness (%)
4.51	13433	1723	21.0	0.035	98.1
3.55	13976	1723	18.4	0.039	97.5
3.09	13770	1723	14.1	0.051	97.4
2.80	13298	1723	10.9	0.064	96.0
2.59	12727	1723	8.55	0.081	94.6
2.42	11913	1723	7.29	0.092	92.3
2.29	11482	1723	6.62	0.099	91.8
2.18	10903	1723	5.97	0.109	87.3
2.09	10085	1723	5.28	0.121	82.6
2.00	8831	1725	4.61	0.132	74.4
Total	120415	17232	10.3	0.052	90.6

1999), the amino-acid sequence homology between these proteins is approximately 30%. Since BLMT shows different activity, determination of its crystal structure is of great interest.

2. Materials, methods and results

The plasmid pUC4KIXX (Barany, 1985), obtained from Pharmacia (Sweden), carries *ble*. Using pUC4KIXX as the DNA template, the *ble* structural gene having *Eco*RI and *Hin*dIII sites at the 5′- and 3′-adjacent regions, respectively, was amplified by PCR. The oligonucleotide primers used in this experiment were 5′-GGGGGAATTCATGACCGACCAAG-CGACGCC-3′ and 5′-GAAGAAGCT-TTCATGAGATGCCTGCAAGCA-3′. The amplified *ble* was subcloned into the *Eco*RI- and *Hin*dIII-digested pKK*trp*. The chimeric plasmid-harboured *Escherichia coli* HB101 was grown at 310 K in 3 l of M9-*cas*amino acid medium (Sambrook *et al.*, 1989) containing 100 μg ml^{-1} ampicillin. 3-Indoleacrylic acid (0.13 m*M*) was added to the culture, which was grown until the exponential phase, for induction of the *ble* gene expression under the control of the *trp* promoter. After cultivation for 24 h, the harvested cells, washed with 20 m*M* Tris–HCl (pH 7.6), were ground with quartz sand and extracted with buffer *A* [20 m*M* Tris–HCl pH 7.6, 30 m*M* NH_4Cl, 10 m*M* magnesium acetate, 6 m*M* 2-mercaptoethanol, 5 m*M* magnesium titriplex (Merck) and 3.45 m*M* phenylmethylsulfonyl fluoride]. The resulting supernatant fluid was collected by centrifugation at 18000*g* for 20 min. Solid ammonium sulfate was added to the supernatant to 20% saturation and the resulting supernatant was collected by centrifugation. BLMT was precipitated by the addition of ammonium sulfate to 40% saturation, dissolution in small volumes of 20 m*M* Tris–HCl (pH 7.6) and dialysis against the same buffer. The dialysate was subjected to a DEAE–Sepharose CL-6B column equilibrated with the same buffer and eluted with 20 m*M* Tris–HCl (pH 7.6) buffer containing 0–1 *M* NaCl in a linear concentration gradient. The fractions containing BLMT, concentrated using ULTRACENT (Tosoh, Japan), were applied to a Sephadex G-75 superfine column for purification to homogeneity. During purification, BLMT was traced by monitoring on SDS–PAGE. The purified BLMT was judged to be pure and homogeneous by the observation of a single band on SDS–PAGE.

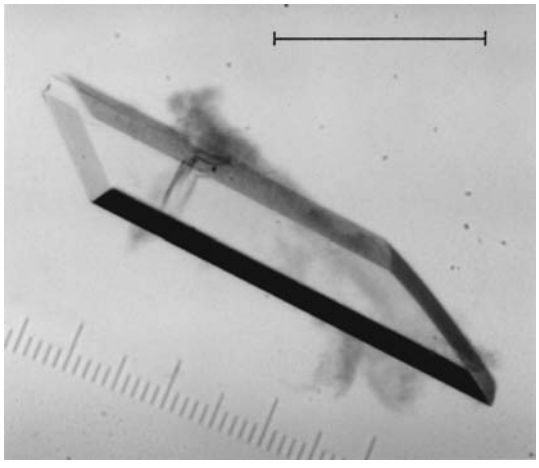

Figure 1
A typical crystal of BLMT. The scale bar is 0.5 mm.

Before crystallization, the protein solution was concentrated by 50% saturated ammonium sulfate precipitation and dissolved in a small volume of 10 m*M* Tris–HCl (pH 7.6).

Crystals of BLMT were grown by vapour diffusion at 298 K using the hanging-drop method (McPherson, 1982). Initial crystallization conditions were determined using sparse-matrix screening (Jancarik & Kim, 1991). Crystallization droplets of 10 μl initial volume were prepared on siliconized glass cover slips suspended over 1.0 ml reservoirs containing 0.2 *M* calcium acetate, 25% PEG 6000 and 0.1 *M* sodium cacodylate pH 6.5. The crystallization droplets consisted of 5 μl (21 mg ml^{-1}) protein solution and 5 μl reservoir solution. After two weeks, crystals of maximum dimensions 0.2 × 0.2 × 1.0 mm were obtained (Fig. 1).

The crystals of BLMT diffract X-rays to at least 2.0 Å resolution. They belong to the orthorhombic space group $C222_1$, with unit-cell dimensions a = 81.56, b = 85.25, c = 78.91 Å. Assuming that the asymmetric unit contains one dimer, the V_m value is calculated to be 2.4 Å^3 Da^{-1}, corresponding to a solvent content of 50% (Matthews, 1968).

The native data collection was carried out using Sakabe's Weissenberg camera (Sakabe, 1983, 1991; Sakabe *et al.*, 1995) at beamline 18B of the Photon Factory, National Laboratory for High Energy Physics, Tsukuba, Japan. All data collection was carried out at room temperature, as the crystals of BLMT were resistant to repeated X-ray radiation. The wavelength was set to 1.00 Å and the crystal was mounted with the c axis parallel to the rotation axis. Weissenberg photographs were recorded on a large-format image plates (IPs; Fuji Film Co. Ltd) with 40 × 80 cm dimensions. The X-ray images recorded on the IPs were read with the corresponding reader for large-format IPs (Sakabe *et al.*, 1995). The diffraction intensities were processed and scaled within 2.0 Å resolution by the *WEIS* program system (Higashi, 1989). A total of 120415 reflections were measured and 17232 independent reflections were obtained with $I > \sigma(I)$, with a merging R value of 0.052 ($R_{merge} = \sum_h \sum_j |I_{hj} - \langle I \rangle_h| / \sum_h \sum_j I_{hj}$, where $\langle I \rangle$ is the mean intensity of a reflection h and I_{hj} is the jth measurement of reflection h). The diffraction data set is 91% complete. Detailed data-collection statistics are given in Table 1.

We have determined the crystal structure of BLMA at 1.5 Å resolution (Kawano *et al.*, 1999). We are in progress of solving the structure of BLMT by molecular replacement with the dimeric BLMA as a search probe. In preliminary analysis, we used a polyalanine model of dimeric BLMA in which the loop regions were omitted as a starting model to obtain a solution of rotation and translation functions using data from BLMT in the resolution range 10–3.5 Å.

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